AD_____

Award Number: DAMD17-01-1-0088

TITLE: Suppressive Role of Androgen-Response Gene Calreticulin in Prostate Cancer

PRINCIPAL INVESTIGATOR: Zhou Wang, Ph.D.

CONTRACTING ORGANIZATION: Northwestern University
Evanston, Illinois 60208-1110

REPORT DATE: December 2004

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	December 2004	Final (1 Jun 2001 - 30 Nov 2004)

4. TITLE AND SUBTITLE
Suppressive Role of Androgen-Response Gene Calreticulin in Prostate Cancer

5. FUNDING NUMBERS
DAMD17-01-1-0088

6. AUTHOR(S)
Zhou Wang, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Northwestern University
Evanston, Illinois 60208-1110

E-Mail: wangz@northwestern.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

Androgens are intimately associated with prostate cancer progression. One of the androgen-response genes encodes calreticulin, a highly conserved protein with demonstrated functions in intracellular Ca^{++} homeostasis, cell adhesion, chaperoning, and gene expression. Our studies showed that calreticulin overexpression is suppressive to tumor growth and metastasis of prostate cancer cells in orthotopic and subcutaneous xenograft tumor models and calreticulin expression is down-regulated in human prostate tumor specimens. Thus, down-regulation of calreticulin in clinical prostate cancer specimens is an important step in prostate cancer progression. Our observations argue that part of androgen-induced gene expression program, such as calreticulin, is inactivated in the progression of prostate cancer, which represents a new concept in prostate cancer biology. In the present study, we have shown that the proline-rich P-domain, which is thought to have lectin-like chaperoning activity, is responsible for the suppression of prostate tumor growth. Our results provide strong basis for further exploring the mechanism by which calreticulin suppresses prostate tumor progression.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Prostate cancer	18
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

Introduction:

Understanding the androgen action pathway in the prostate has clinical significance.

Androgen plays an important role in the development and progression of prostate cancer (Bosland, 1992; Carter and Coffey, 1990; Kozlowski and Grayhack, 1991; Lee et al., 1995). Understanding the androgen action pathway in the prostate will provide insights into the mechanisms by which androgen impacts the pathogenesis of prostate cancer, and may lead to more effective approaches for its prevention and treatment. The androgen action pathway here is defined as a cascade of molecular and cellular events triggered by androgen manipulation leading to cell proliferation, apoptosis, and/or differentiation.

Androgen controls homeostasis of the prostate.

As shown in Table 1, androgen stimulates proliferation and differentiation in a regressed prostate but not in a fully-grown prostate. On the other hand, androgen ablation induces massive apoptosis and rapid dedifferentiation in a fully-grown prostate, but has little or no effect on a regressed prostate. These observations suggest that in the regrowth process of a regressed prostate, androgen replacement stimulates and then nullifies proliferation, establishes apoptotic potential while inhibiting apoptosis, and induces and maintains differentiation. The molecular mechanisms by which androgen controls prostate regrowth remain largely unclear.

Table 1. The impact of androgen manipulation on the regressed prostate and the normal prostate.

Androgen	Regressed Prostate	Fully-Grown Prostate
+	Proliferation & Differentiation	No Significant Change
-	No Significant Change	Apoptosis & Dedifferentiation

+ represents androgen replacement and – represents androgen ablation or administration of anti-androgens. Differentiation is defined as the expression of prostate-specific markers. Dedifferentiation is defined as loss of the expression of prostate-specific markers.

Androgen action is mediated through androgen-response genes including calreticulin.

The dramatic influence of androgen on the prostate is mediated through androgen receptor (AR). AR is a ligand-dependent transcription factor that regulates the expression of androgen-response genes, either directly or indirectly (Mainwaring, 1977; Zhou et al., 1994). Thus, androgen-response genes should mediate AR downstream events leading to cellular and morphological changes in the prostate during androgen manipulation.

To study the androgen action pathway, we have searched for androgen-response genes on the basis of their induction during the initial regrowth of the regressed ventral prostate in 7-day castrated rats using a highly sensitive PCR-based cDNA subtraction method (Wang and Brown, 1991; Wang et al., 1997). Our search has identified 25 genes that are up-regulated by androgen and 4 genes that are down-regulated by androgen in the ventral prostate of a 7-day castrated rat.

One of the androgen-response genes encodes calreticulin. Our recent studies suggest that calreticulin has significant growth suppressive role in prostate cancer and its expression is down-regulated in prostate cancer cells. These observations argue that part of the androgen action pathway, which is growth suppressive, is down-regulated in prostate cancer pathogenesis.

Calreticulin is a multi-functional Ca^{++} binding protein.

Calreticulin is an evolutionarily conserved major Ca^{++} binding protein in endoplasmic reticulum ER (Krause and Michalak, 1997; Michalak et al., 1992; Sontheimer et al., 1995). Calreticulin has been implicated in the regulation of a variety of cellular functions including the regulation of intracellular Ca^{++} homeostasis (Bastianutto et al., 1995; Liu et al., 1994; Mery et al., 1996; Zhu and Wang, 1999), cell adhesion (Coppolino et al., 1995; Dedhar, 1994; Fadel et al., 1999; Opas et al., 1996), steroid-mediated gene regulation (Burns et al., 1994; Dedhar et al., 1994; Michalak et al., 1996), chaperone activity (Nauseef et al., 1995; Peterson et al., 1995; Vassilakos et al., 1998; Zapun et al., 1998), Zn^{++} binding, and rubella virus RNA binding. Calreticulin gene knockout mice are embryonic lethal because calreticulin is essential for cardiac development (Mesaeli et al., 1999).

Calreticulin consists of 400 a.a. residues after the N-terminal signal sequence is removed by posttranslational processing (Baksh and Michalak, 1996). It has a calculated MW of 46 kd and an apparent MW of 60 kd in SDS PAGE gel. There is a KDEL ER retention sequence at the C-terminal end of calreticulin. Calreticulin consists of at least 3 structural/functional domains (Fig. 1).

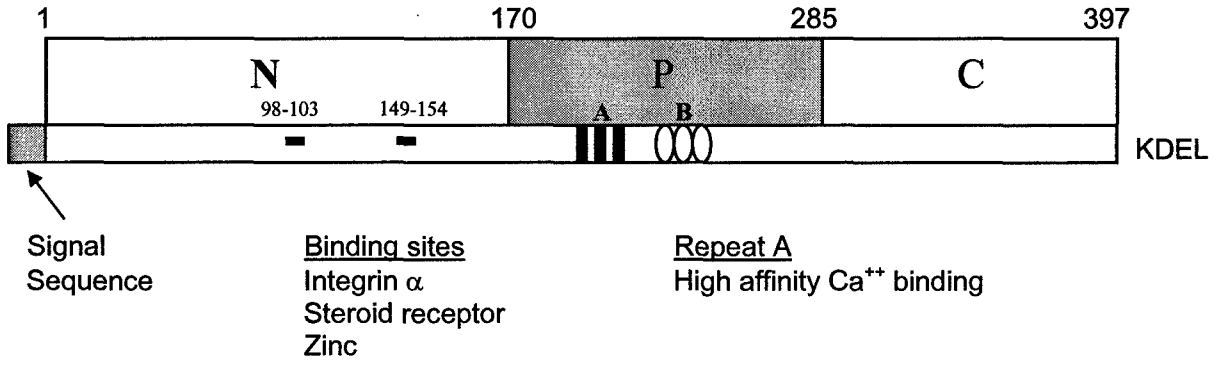


Fig. 1. The domain structure of calreticulin (Baksh and Michalak, 1996). The N-domain (aa 1-170) of calreticulin is the most conserved domain in evolution and does not bind to Ca^{++}. The N-domain forms a globular domain with 2 regions of short α-helices at residues 98-103 and 149-154, which are responsible for the binding to integrin α and the DNA binding domain of steroid receptors. The P-domain (aa 171-285) is proline-rich and contains two sets of repeats (Repeat A and Repeat B). Repeat A consists of three repeats of aa sequence PXXIXDPDAXKPEDWDE and is believed to be responsible for high affinity (Kd = 1.6 uM) and low capacity (1 Ca^{++}/protein) Ca^{++} binding to calreticulin. Repeat B consists of three aa sequence GXWXPPXIXNPXYX and is predicted to have a rigid turn structure separating the globular head of the protein from the acidic tail. The C-domain (aa 286-397) is highly acidic and negatively charged. This large stretch of negatively charged residues binds Ca^{++} with low affinity (Kd = 0.3-0.2 mM) and high capacity (~25 Ca^{++}/protein). These high capacity low affinity Ca^{++} binding sites have led to the hypothesis that calreticulin is involved in luminal Ca^{++} storage. The C-domain in calreticulin has the most divergent aa sequence among different species.

Calreticulin is abundantly expressed and regulated by androgen in prostate epithelial cells.

We became interested in calreticulin because it was identified in our search for androgen-response genes in the rat ventral prostate (Wang et al., 1997; Zhu et al., 1998). Androgen ablation by castration rapidly down-regulates calreticulin at both mRNA and protein levels for more than 10-fold. In contrast, androgen replacement rapidly restores the expression of calreticulin in the regrowth of the castrated prostate. Northern blot analysis of tissue-

specificity of calreticulin expression showed that calreticulin expression in the prostate is much more abundant than its expression in any other surveyed organs including liver, kidney, brain, heart, muscle, and seminal vesicles. In situ hybridization and immunohistochemistry studies demonstrated that calreticulin is an intracellular protein in the epithelial cells of the prostate. Calreticulin expression in human epithelial cells is also regulated by androgens, suggesting that calreticulin regulation by androgen is conserved in evolution (Zhu et al., 1998; Zhu and Wang, 1999).

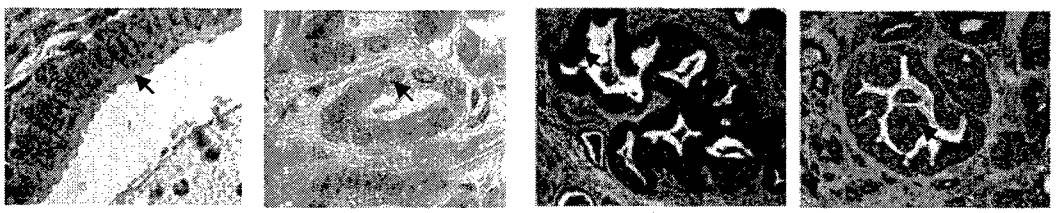
Body:

Task 1: Test the hypothesis that calreticulin down-regulation is more frequent in high Gleason grade prostate tumors (months 1-36).

a. Collect clinical specimens (months 1-36).
b. Calreticulin expression will be determined by IHC. The timing, magnitude, and frequency of calreticulin down-regulation in clinical prostate cancer specimens will be determined (months 1-36).
c. Statistical analysis will be applied to determine whether calreticulin down-regulation correlates with the Gleason grade (months 30-36).

Calreticulin expression is down-regulated in human prostate cancer specimens.

Expression of calreticulin in 21 hormone naïve clinical prostate specimens from radical prostatectomy was examined by immunohistochemistry (IHC) using an anti-calreticulin antibody (Zhu et al., 1998). These specimens contain benign regions, tumors, and/or high grade prostatic intraepithelial neoplasia (HGPIN). Calreticulin expression was down-regulated, to various extent, in 4 out of 11 HGPIN, 4 out of 10 Gleason 3 prostate tumors, and 2 out of 3 Gleason 4 prostate tumors. Examples of typical down-regulation are shown in Fig. 2. No calreticulin down-regulation was observed in benign prostatic epithelial cells in all of the specimens. These observations suggest that calreticulin down-regulation is more frequent in tumors with high Gleason score, which is associated with poor prognosis (Gleason and Mellinger, 1974). One clinically significant question is whether the cancer cells with loss of calreticulin expression will become highly metastatic and life-threatening.



A) Benign **B) Gleason 3** **C) Benign** **D) HGPIN**

Fig. 2. IHC studies of calreticulin expression in clinical prostate tumor specimens. One specimen containing both benign prostate (A) and Gleason 3 cancerous prostate (B) was stained with anti-Crt antibody and hemotoxylin as described previously (Zhu et al., 1998). Another specimen containing both benign prostate (C) and HGPIN (D) was stained with anti-Crt antibody but without hemotoxylin. Secondary antibody alone did not stain the section (Results not shown). The benign and cancerous epithelial cells are marked with arrows.

Table 1. Calreticulin immunostaining intensity in 21 human prostate tumor specimens.

Intensity	Benign (n=21)	HGPIN(n=11)	Gleason 3 (n=10)	Gleason 4 (n=3)
+	0	0	10%	33.3%
++	0	36.4%	30%	33.3%
+++	100	63.6%	60%	33.3%

The intensity of the staining was determined using specimens stained with anti-calreticulin without hemotoxylin. +++ represents normal level staining; ++ represents moderate down-regulation; + represents barely detectable expression of calreticulin.

Recently, we have extended our studies using prostate cancer tissue array. The arrays contained 124 tissue cores derived from 42 patients. The staining intensity was graded from 0 to 4 in a blinded fashion as described by Grizzle et al (Grizzle et al., Immunohistochemical Evaluation of Biomarkers in Prostatic and Colorectal Neoplasia). The down-regulation of calreticulin was reproduced and the extent of down-regulation correlates with Gleason grade (Fig. 3).

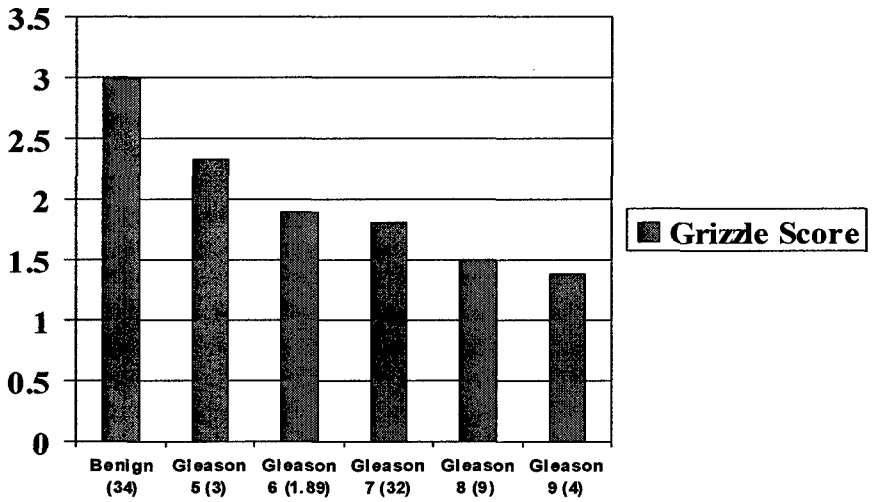


Fig. 3. Calreticulin staining intensity of benign or cancerous human prostate tissue specimens in tissue microarray. The Gleason scores of tumor specimens were indicated.

Task 2: Determine the functional domains and motifs of calreticulin in inhibiting anchorage-independent growth (months 1-36).

a. Deletion mutants will be generated to map domain(s) essential for inhibiting anchorage-independent growth of prostate cancer cells (months 1-24).
b. Substitution mutants will be generated to map essential motifs (months 1-24).
c. The impact of calreticulin mutants on the growth of PC3 prostate cancer cells in soft agar will be tested to identify essential amino acid sequences in calreticulin (months 25-36).

Calreticulin markedly inhibits anchorage-independent growth of prostate cancer cells.

We cloned calreticulin cDNA into the pcDNA3.1/Hygro(+) vector (Invitrogen) to generate calreticulin (crt) expression vector pcDNA3.1/crt. The pcDNA3.1/crt was then stably transfected into PC3, a highly aggressive androgen-independent human prostate cancer cell line. Endogenous calreticulin level is very similar between PC3 clones transfected with empty vector and parental cells (Fig. 4). PC3 cells transfected with pcDNA3.1/crt express calreticulin at varying levels (2.5-10X) above the endogenous calreticulin level of the parental cell line. The highest calreticulin expression levels achieved in PC3 cells are similar to the calreticulin level in the intact rat ventral prostate (Fig. 4) and the calreticulin level in the intact rat ventral prostate represents a physiologically relevant level.

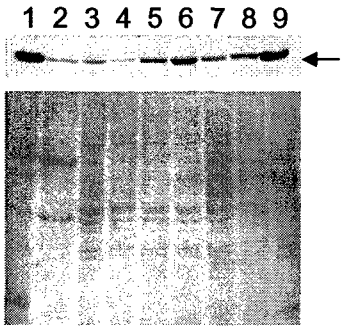


Fig. 4. The expression of calreticulin in parental, empty vector transfected, and pcDNA3.1/crt transfected PC3 and LNCaP cells. Lane 1 = Normal rat ventral prostate; Lane 2 = 7-day castrated rat ventral prostate; Lane 3 = Parental PC3; Lane 4 = Empty vector transfected PC3 (Mock); Lane 5 = pcDNA3.1/crt transfected PC3 clone 35 (Crt35); Lane 6 = pcDNA3.1/crt transfected PC3 clone 59 (Crt59); Lane 7 = Parental LNCaP; Lane 8 = Empty vector transfected LNCaP; and Lane 9 = pcDNA3.1/crt transfected LNCaP. The arrow indicates calreticulin. The loading of total protein was visualized by Ponceau-S staining. The Western blot represents one example of 4 experiments.

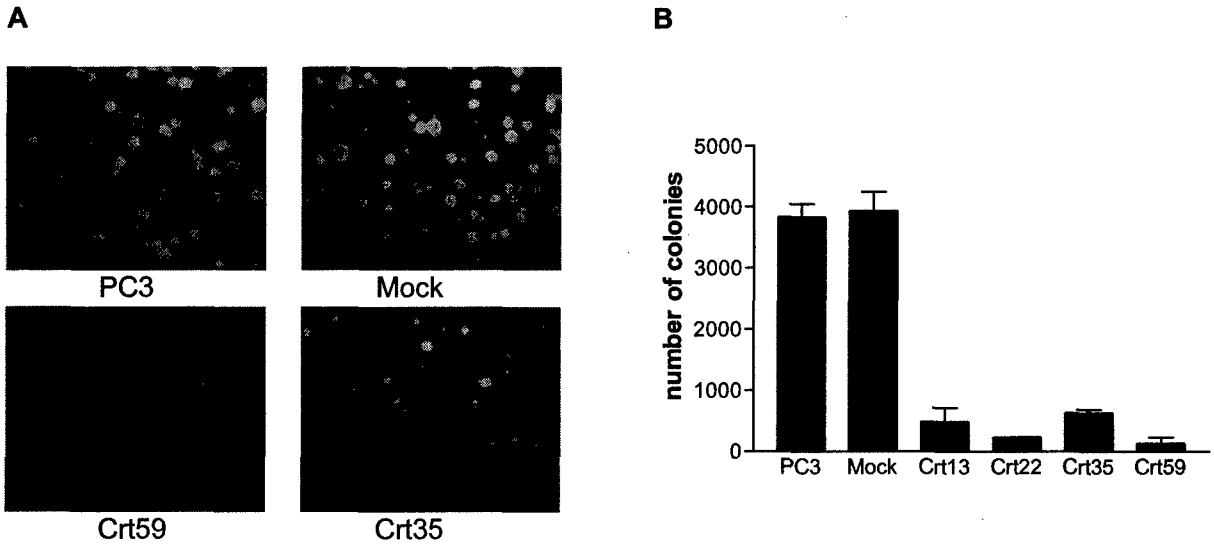


Fig. 5. The effect of calreticulin overexpression on colony formation of PC3 cells in soft agar assay. A) The colony formation of PC3, Mock, Crt 35 and Crt 59 in soft agar. The soft agar assay was conducted in 6-well plates. The bottom agar is 2 ml 0.6% noble agar containing 1X RPMI 1640 medium supplemented with10% FBS. After the preparation of bottom agar, 1 ml of 0.3 % top agar containing 5,000 cells and 1X RPMI 1640 medium supplemented with10% FBS was added to form the top layer. The assay for each PC3 subline was carried out in duplicate and was repeated at least 3 times. B) The quantification of colony formation PC3, Mock, Crt13, Crt22, Crt 35 and Crt 59. Colonies with greater than 125 um in diameter were counted in the quantification. Error bars represent standard error means (SEM).

Overexpression of calreticulin significantly inhibited the size and number of the PC3 colonies in soft agar assay (Fig. 5). Dramatic inhibition of soft agar colony formation was reproducibly observed in all four PC3 sublines with overexpressed calreticulin. Clones Crt59 and Crt35 express calreticulin at a level about 10-fold and 2.5-fold above the endogenous level respectively. Clone Crt13 and Crt22 express calreticulin about 5-fold above endogenous level. The degree of colony inhibition appears to correlates with the level of calreticulin overexpression in the four PC3 sublines. Fig. 5 shows that clone Crt59 (10X endogenous) forms less colonies in soft agar than that in clone Crt35 (2.5X endogenous). Colony formation in clone Crt13 and Crt22 (5X endogenous) is between clones Crt59 and Crt35 (Fig. 5B).

As expected, overexpression of calreticulin also markedly inhibited anchorage-independent growth of TSU prostate cancer cells (Result not shown). This suggests that calreticulin inhibition of anchorage-independent growth is a general phenomenon in prostate cancer cells. Anchorage-independent growth in soft agar correlates with the metastatic potential of cancer cells (Cifone and Fidler, 1980; Li et al., 1989). Thus, this observation suggests that calreticulin has the potential to suppress metastasis of prostate cancer cells *in vivo*.

Construction of mutant calreticulin expression vectors.

To prepare for mechanistic studies, we have constructed a series of mutant calreticulin expression vectors. As illustrated in Fig. 1, calreticulin consists of 3 structural domains, N, P, and C. Dr. Marek Michalak has kindly provided us with HA tagged wild-type rabbit calreticulin (Rb Crt-HA) and 3 HA-tagged mutants (Rb N-HA, Rb N+P-HA, and Rb P-HA). We have made additional HA-tagged mutant calreticulin expression vectors. In addition, we have also generated GFP-tagged mutant calreticulin expression vectors (Fig. 6), which will allow us to conveniently determine the intracellular localization of various calreticulin mutants.

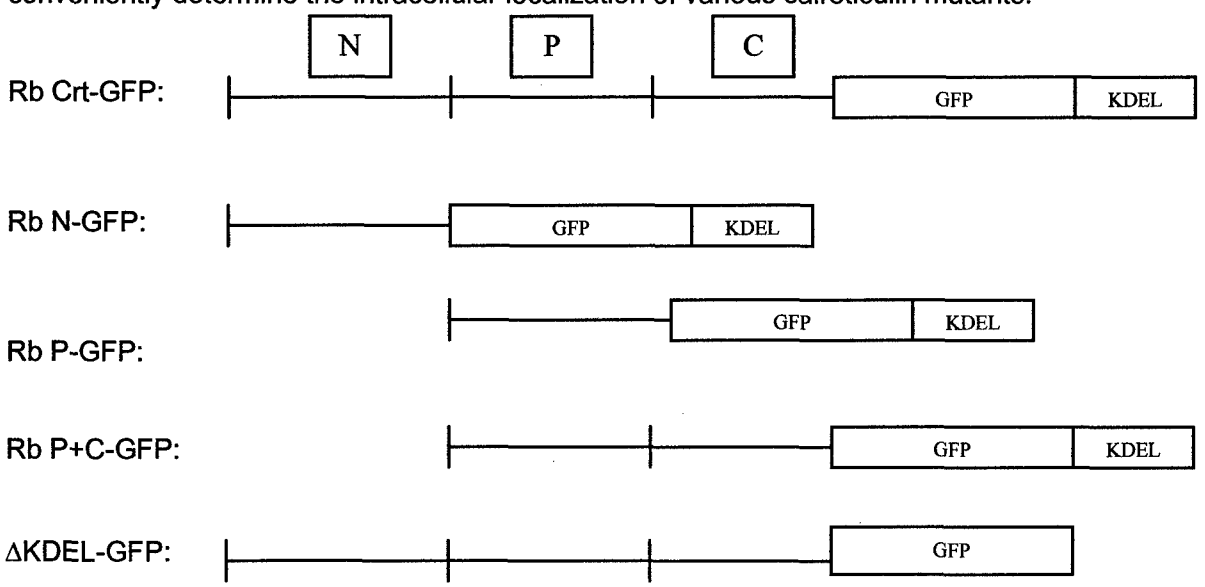


Fig. 6. Green fluorescent protein (GFP)-tagged wild-type calreticulin and calreticulin mutants. The rabbit calreticulin was used in the mutant construction. N, P, and C stand for N-domain, P-domain, and C-domain respectively (See Fig. 1 for details). All of the constructs were verified by sequencing analysis. All of the constructs have the signal peptide sequence at their N-terminus.

P and C domain of calreticulin are important for endoplasmic reticulum localization of calreticulin.

In the course of our investigation, we realized one important issue regarding calreticulin mutants - whether mutations will affect intracellular localization of calreticulin. As expected, wild-type calreticulin is colocalized with PDI, an ER marker (Fig. 7). If a mutation altered the intracellular localization of calreticulin, it is likely to have a dramatic impact on calreticulin function. It will be important to for us to conduct functional analysis on calreticulin mutants that are still localized in endoplasmic reticulum. Thus, we have carried out extensive analysis on the intracellular localization of various GFP-tagged calreticulin mutants. The surprising finding is that the KDEL ER-retention signal is not necessary for proper calreticulin localization (Fig. 8). The P and C domain contains signals for ER localization (Fig. 8), which was not expected. These findings will allow us to focus on mutants containing P and/or C domain.

Crt-GFP colocalizes with an ER marker (PDI)

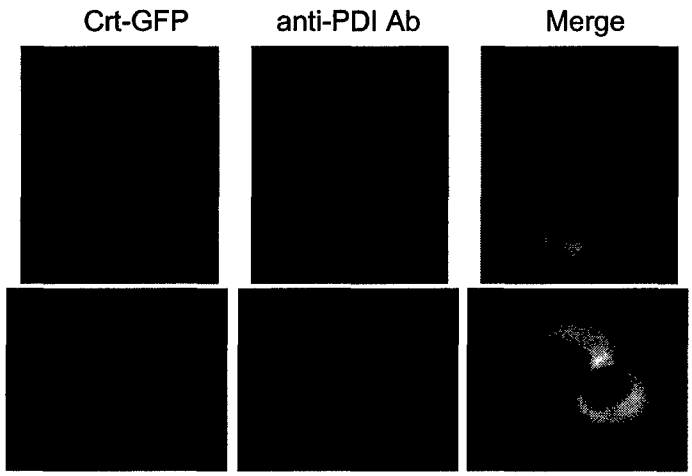


Figure 7. Co-localization of Crt-GFP with protein disulfide isomerase (PDI), an ER-resident protein. Crt-GFP/pcDNA3.1 was transiently-transfected into PC3 cells using Fugene 6.0. Cells were plated onto coverslips and fixed in 4% paraformaldehyde. Immunofluorescence was performed using an α-PDI antibody (Stressgen).

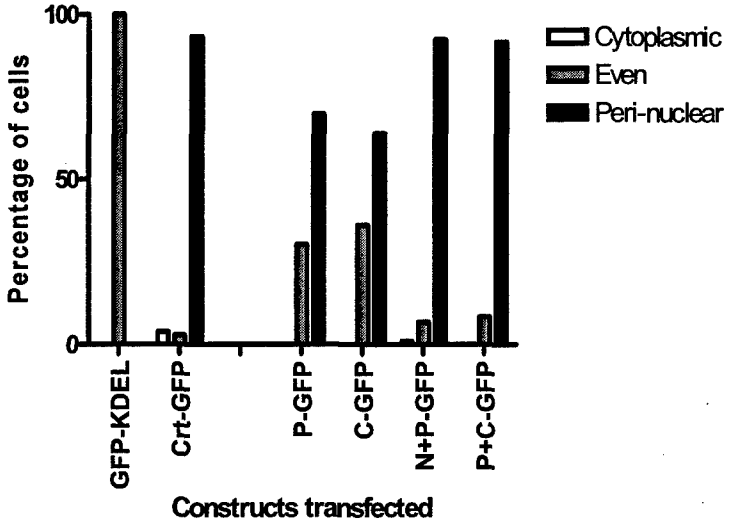


Figure 8. Quantification of localization of Crt-GFP and domain mutants fused to GFP. Crt and its domain mutants fused to GFP were transiently transfected into PC3 cells using Fugene 6.0. After 24 hrs, the number of cells showing peri-nuclear compartment localization was counted (minimum of 100 cells per sample).

In addition, we have generated 8 substitution mutants in the conserved domains (Fig. 9). These substitution mutants will allow us to define the functional significance of the conserved domains.

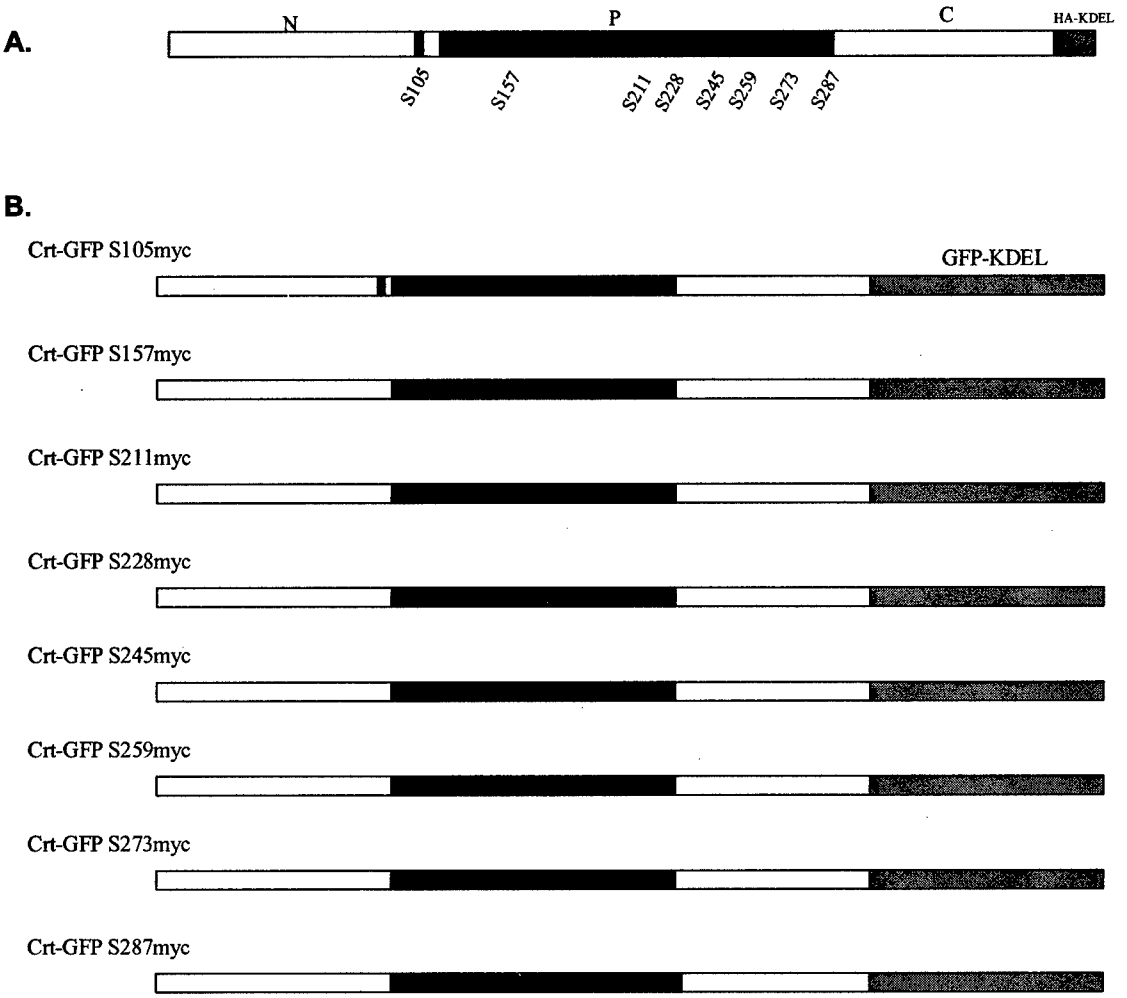


Fig. 9. Substitution mutants in 8 conserved calreticulin domains. A. Positions of 8 conserved amino acid stretches (10-11 amino acid residues in length). These positions in calreticulin were replaced with the myc epitope tag individually. **B.** GFP-tagged substitution mutants. Various substitution mutants were fused to GFP using a PCR-based approach. All the mutants were verified by sequencing.

We have selected multiple stable clones to assess the effect of mutant calreticulin overexpression on anchorage-independent growth in soft agar. The results were disappointing and the clonal variation prevented us from making meaningful interpretations. To overcome the clonal variation problem, we have tested a number of alternatives. One approach, clonogenic assay, worked well for in vitro functional study. Fig. 10 showed that transfection with calreticulin expression vectors, relative to the control vectors, reproducibly inhibited the colony formation in both PC3 and LNCaP cells. The inhibition is about 2-fold in PC3 cells and 5- to 20-fold in LNCaP cells. We have tested the ability of various calreticulin mutants to inhibit colony formation in PC3 cells. The results showed that calreticulin mutants containing P-domain

inhibited colony formation whereas the mutants without P-domain did not. Our finding indicates that the P-domain is required for inhibition of prostate tumor cell growth.

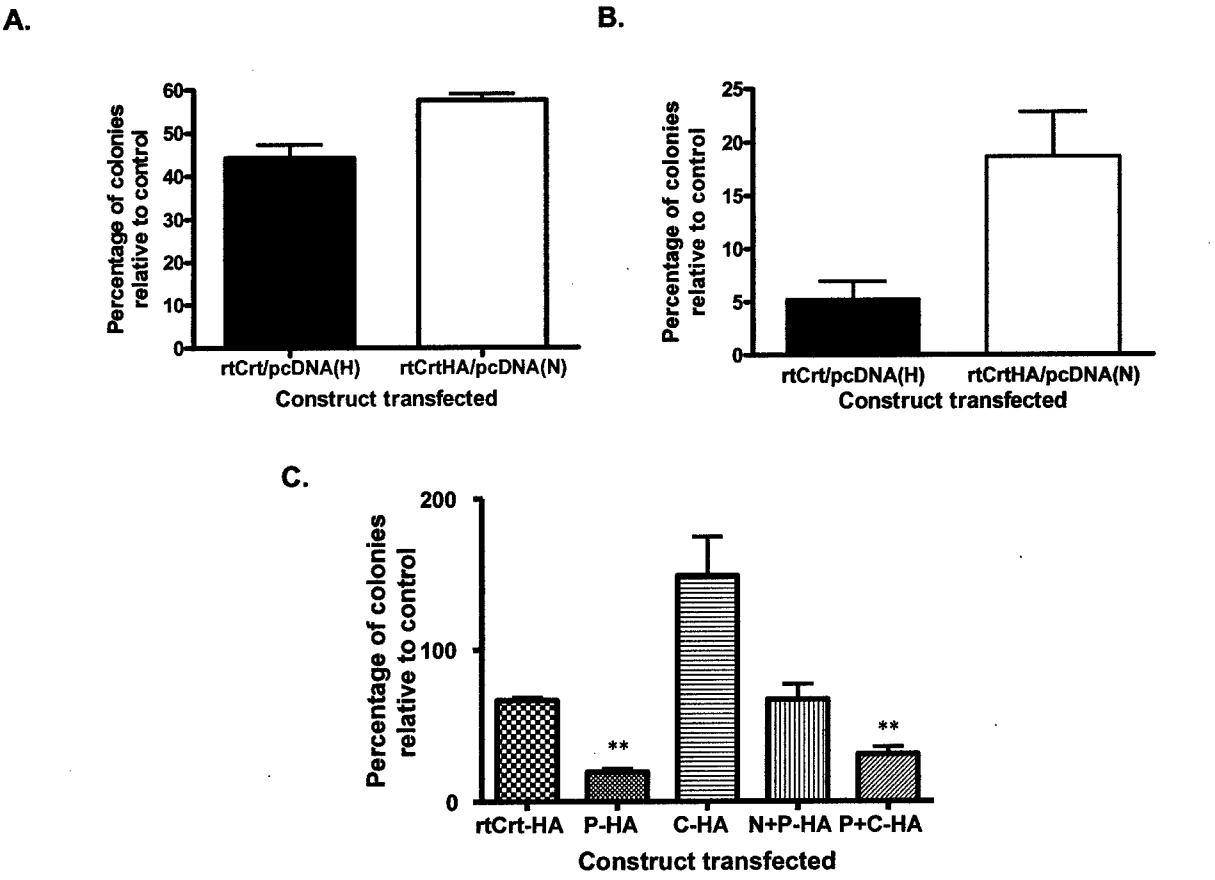


Fig. 10. Effect of wild-type and mutant calreticulin overexpression on colony formation. **A.** Inhibition of PC3 colony formation by overexpression of untagged rat calreticulin (rtCrt/pcDNA) or HA-tagged rat calreticulin (rtCrtHA/pcDNA). Controls are the empty vectors. **B.** Inhibition of LNCaP colony formation by overexpression of untagged rat calreticulin (rtCrt/pcDNA) or HA-tagged rat calreticulin (rtCrtHA/pcDNA). Controls are the empty vectors. **C.** Inhibition of PC3 colony formation by overexpression of HA-tagged rat calreticulin mutants. P-HA: P-domain tagged with HA; C-HA: C-domain tagged with HA; N+P-HA: N- and P- domain tagged with HA; P+C-HA: P- and C-domain tagged with HA. Controls are the empty vectors. PC3 or LNCaP cells were transfected with indicated expression vector or empty vectors. After transfection, the cells were subject to hygromycin (H) or neomycin (N) selection in 10 cm plates for about 3 weeks. The number of colonies was counted and the experiments were repeated at least 3 times.

Task 3: Study the role of calreticulin in prostate tumor growth and metastasis *in vivo* in tumor xenografts (months 1-36).

a. The effect of wild-type calreticulin on the growth and metastasis of subcutaneous and orthotopic xenograft prostate tumors will be studied in 32 nude mice (months 1-12).
b. The effect of calreticulin deletion mutants on the growth and metastasis of subcutaneous and orthotopic xenograft prostate tumors will be studied in 56 nude mice (months 13-24).

c. The effect of calreticulin substitution mutants on the growth and metastasis of subcutaneous and orthotopic xenograft prostate tumors will be studied in 56 nude mice (months 25-36).

Restoration of calreticulin expression inhibits metastasis of rat Dunning AT3.1 xenograft tumors in nude mice.

Dr. Allen Gao has generously provided us with the Dunning rat prostate cancer cell lines including the G, AT1, AT2, AT3.1, AT6.1, and Mat-Lylu, which were derived originally from the rat dorsolateral prostate. Calreticulin is down-regulated in all of the above prostate cancer cell lines (Result not shown). AT3.1 cell line was chosen as a model in this study for testing the impact of calreticulin overexpression on metastasis because subcutaneous AT3.1 xenogroft tumors readily generate countable macromatastasis in lungs of the host nude mice.

AT3.1 cells were stably transfected with the calreticulin expression vector pcDNA-Crt. Several AT3.1 sublines expressing ectopic calreticulin were established and the level of calreticulin expression in the stable lines are similar to the dorsal and lateral prostates of the rat (Data not shown). The number of lung metastases was inhibited in the nude mice bearing xenografted tumors of these sublines (Fig. 11A). In contrast, calreticulin overexpression did not inhibit the wet weight of primary tumors (Fig. 11B). These observations indicate that normal levels of calreticulin suppress metastasis rather than the growth of prostate cancer *in vivo*.

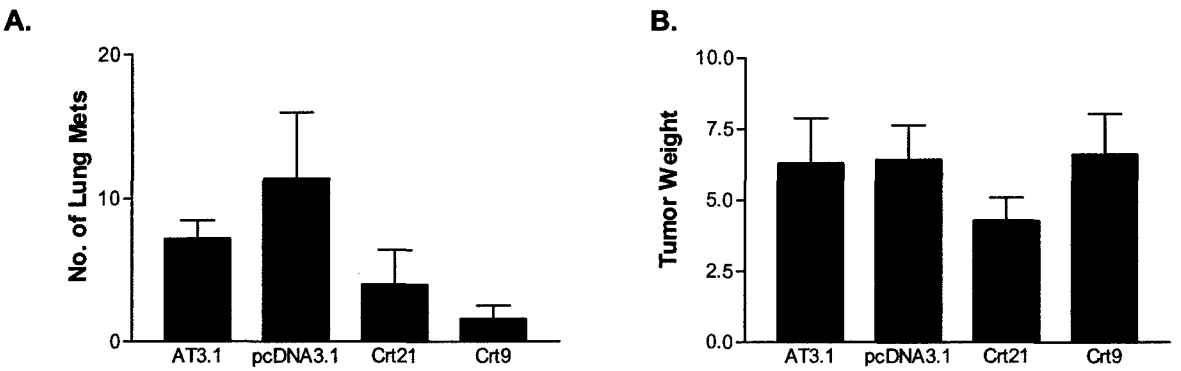


Fig. 11. A. Effect of calreticulin overexpression on AT3.1 xenograft tumor metastasis in nude mice. The parental AT3.1 (AT3.1), empty vector transfected AT3.1 (pcDNA3.1), and calreticulin expression vector transfected AT3.1 cells (Crt9, Crt21) were used to establish subcutaneous xenograft tumors in male nude mice. The cultured cancer cells were trypsinized and the single cell suspension was prepared in RPMI 1640 containing 10% FBS. Approximately 400,000 cells in 0.1 ml volume were injected subcutaneously into 4-6 week old male athymic nude mice, with 5 mice each subline (Charles River Laboratories, Frederick, MD) in the both flank. Eighteen days after injection, the tumor bearing mice were sacrificed according to a procedure approved by institutional ACUC committee. The primary tumors were excised and weighted. The lungs were fixed in Bouin's solution and lung macro-metastases were scored under a dissection microscope. B. Effect of calreticulin overexpression on the weight of AT3.1 primary xenograft tumors. The wet weight of each primary tumor was determined immediately after resection.

We have also studied the effect of calreticulin overexpression on PC3 orthotopic xenograft tumor growth. We have established new PC3 sublines overexpressing rat calreticulin tagged with HA epitope (rtCrtHA) to make sure the stable PC3 sublines are at low passage. We

verified the overexpression of calreticulin in the PC3 sublines (Fig. 12A) and reproduced our finding in Fig. 5 that calreticulin overexpression inhibited the growth of transfected PC3 cells in soft agar (Fig. 12B & C). We evaluate the growth of orthotopically implanted mock and calreticulin transfected PC3 sublines in nude mice (Fig. 12D). The results argue that calreticulin inhibits PC3 orthotopic xenograft tumor growth.

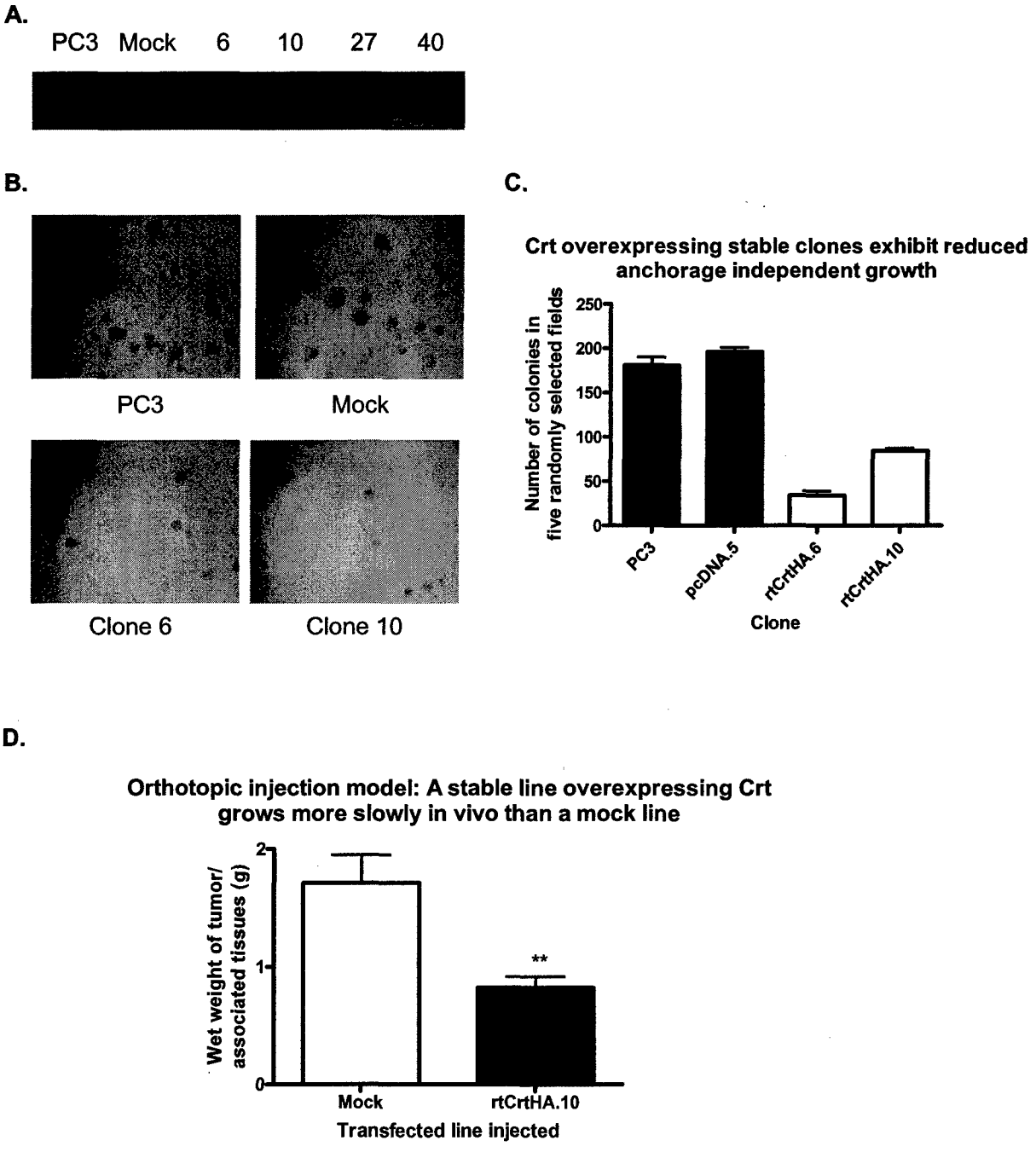


Fig. 12. Effect of calreticulin (Crt) overexpression on the growth of PC3 cells in soft agar and in orthotopic xenograft tumors. A. Calreticulin expression in parental PC3, empty-vector

transfected PC3 cells (Mock), and calreticulin-transfected stable PC3 subline 6, 10, 27 and 40. Western blot analysis was performed using an anti-calreticulin antibody. B. The effect of calreticuin overexpression on the growth of PC3 cells in soft agar. The method was the same as described in Fig. 5 legend. C. Quantification of colony formation of PC3 cells in B. D. The effect of calreticulin overexpression on PC3 orthotopic xenograft tumor growth in nude mice. The mock, pcDNA.5 and the rtCrtHA.10 was orthotopically injected into the dorsal lobe under the capsule. One million cells in 20 ul were used in each injection and 15 mice were used for each cell line. The mice were analysis 40 days after the injection. The wet weight of prostate and associated urethra was determined. The difference between mock and rtCrtHA.10 is statistically significant ($p < 0.005$).

To accomplish proposed Specific Aim 3b and 3c, we have repeatedly tried to obtain stable sublines transfected with various calreticulin mutants. These experiments are time consuming. Unfortunately, the clonal variations are huge with these mutant calreticulin constructs in soft agar assays. We have tried alternative approaches. One alternative approach is using tet-inducible expression vector. Although we obtained tet-inducible calreticulin expression, the induced expression level is very low and less than endogenous calreticulin level. Thus, the stable clones with tet-inducible expression of calreticulin were not useful in functional studies. In addition, we have tried to use adenoviral expression vector to deliver calreticulin, which should avoid clonal variation problem. However, infection of adenovirus carrying calreticulin causes apoptosis of the infected cells. We have sequence verified the adenoviral vector and the calreticulin sequence was correct. In addition, Western blot detected the expression of adenoviral delivered calreticulin expression. At present, we are unable to explain the above observation. Our current studies indicate that Specific Aims 3b and 3c are not feasible.

Key Research Accomplishments:

1. Calreticulin expression is down-regulated in human prostate cancer specimens.

Our hypothesis states that calreticulin is suppressive to prostate cancer malignancy. If calreticulin suppression of prostate cancer progression is important *in vivo*, the expression of calreticulin should be down-regulated in clinical prostate cancer specimens. Our studies provided evidence for the down-regulation of calreticulin *in vivo* in clinical specimens, which is critical to this project.

2. Construction of calreticulin substitution mutant expression vectors.

Substitution mutagenesis is a powerful approach for elucidating the motif(s) that is critical for protein function(s). To identify motif(s) in calreticulin responsible for the suppression of anchorage-independent growth and/or metastasis of prostate cancer cells, we have generated 8 calreticulin substitution mutants either tagged with GFP. These mutants will provide insights into the mechanism by which calreticulin suppresses metastasis.

3. P and C domains in calreticulin are critical for proper intracellular localization of calreticulin.

To facilitate our functional studies proposed in specific aim 3, it is important to assess the impact of various mutations on calreticulin intracellular localization. Our data showed that the P and/or C domain containing calreticulin mutants are capable of localizing to endoplasmic

reticulum. We focused on calreticulin mutants with endoplasmic reticulum localization in subsequent studies.

4. P domain is required for calreticulin to inhibit prostate tumor cell growth.

Our result showed that calreticulin mutant constructs containing P domain can suppress colony formation of both transfected PC3 and LNCaP cells. In contrast, calreticulin mutants with P domain deleted do not inhibit colony formation of PC3 and LNCaP cells. Since P domain is thought to have chaperone activity, the chaperone function of calreticulin is likely responsible for inhibiting prostate tumor growth.

5. Calreticulin overexpression inhibits anchorage-independent growth of PC3 cells.

We have demonstrated that calreticulin overexpression markedly inhibited anchorage-independent growth of PC3 cells in soft agar assays. The levels of calreticulin overexpression correlate with the degree of the inhibition. Since the ability of cancer cells to grow in soft agar is often associated with their metastatic potential, calreticulin overexpression may suppress tumor metastasis.

6. Calreticulin suppresses metastasis in rat Dunning AT3.1 prostate tumor model.

Rat Dunning AT3.1 prostate tumor cell line is a widely used model for studying prostate tumor metastasis. Thus, this cell line was used to test the hypothesis that calreticulin overexpression is suppressive to tumor metastasis. Our studies indicate that AT3.1 cells with calreticulin overexpression generated less lung metastasis relative to the parental and empty vector-transfected AT3.1 cells.

7. Calreticulin suppresses the growth of orthotopically implanted PC3 xenograft tumors in nude mice.

PC3 cell line is a commonly used human prostate cancer cell line. Orthotopic implantation allows the implanted tumor cells grow in the prostatic environment. Our data argue that calreticulin suppresses the growth of orthotopic PC3 xenograft tumors. Thus, calreticulin is potentially inhibitory to primary prostate tumor growth.

Reportable Outcomes:

1. A manuscript entitled "Calreticulin suppresses prostate tumor growth via proline-rich P domain" is in preparation.
2. A manuscript entitled "Identification and characterization of domains responsible for endoplasmic reticulum localization of calreticulin" is in preparation.

Conclusions:

Our studies have indicated that calreticulin overexpression is suppressive to anchorage-independent growth and metastasis of prostate cancer cells and calreticulin expression is down-regulated in human prostate tumor specimens. We further showed that calreticulin overexpression inhibited the growth of orthotopic PC3 xenograft tumors. We have discovered that the P and C domain, rather than the KDEL signal, is critical for calreticulin to localize to endoplasmic reticulum. Furthermore, we showed that P-domain is required for calreticulin to inhibit prostate cancer cell growth in clonogenic assay. This finding is mechanistic and future studies should focus on the P-domain mediated biological activities.

References:

Baksh, S., and Michalak, M. (1996). Basic characteristics and ion binding to calreticulin. In Calreticulin, M. Michalak, ed. (Austin, Texas, R.G. Landers), pp. 11-30.

Bastianutto, C., Clementi, E., Codazzi, F., Podini, P., De Giorgi, F., Rizzuto, R., Meldolesi, J., and Pozzan, T. (1995). Overexpression of calreticulin increases the Ca2+ capacity of rapidly exchanging Ca2+ stores and reveals aspects of their lumenal microenvironment and function. Journal of Cell Biology *130*, 847-855.

Bosland, M. (1992). Animal models for the study of prostate carcinogenesis. [Review]. Journal of Cellular Biochemistry - Supplement *16H*, 89-98.

Burns, K., Duggan, B., Atkinson, E. A., Famulski, K. S., Nemer, M., Bleackley, R. C., and Michalak, M. (1994). Modulation of gene expression by calreticulin binding to the glucocorticoid receptor. Nature *367*, 476-480.

Carter, H., and Coffey, D. (1990). The prostate: an increasing medical problem. Prostate *16*, 39-48.

Cifone, M. A., and Fidler, I. J. (1980). Correlation of patterns of anchorage-independent growth with *in vivo* behavior of cells from a murine fibrosarcoma. Proceedings of the National Academy of Sciences of the United States of America *77*, 1039-1043.

Coppolino, M., Leung-Hagesteijn, C., Dedhar, S., and Wilkins, J. (1995). Inducible interaction of integrin alpha 2 beta 1 with calreticulin. Dependence on the activation state of the integrin. Journal of Biological Chemistry *270*, 23132-23138.

Dedhar, S. (1994). Novel functions for calreticulin: interaction with integrins and modulation of gene expression? Trends in Biochemical Sciences *19*, 269-271.

Dedhar, S., Rennie, P. S., Shago, M., Hagesteijn, C. Y., Yang, H., Filmus, J., Hawley, R. G., Bruchovsky, N., Cheng, H., Matusik, R. J., and et al. (1994). Inhibition of nuclear hormone receptor activity by calreticulin. Nature *367*, 480-483.

Fadel, M. P., Dziak, E., Lo, C. M., Ferrier, J., Mesaeli, N., Michalak, M., and Opas, M. (1999). Calreticulin affects focal contact-dependent but not close contact-dependent cell-substratum adhesion. Journal of Biological Chemistry *274*, 15085-15094.

Gleason, D. F., and Mellinger, G. T. (1974). Prediction of prognosis for prostatic adenocarcinoma by combined histological grading and clinical staging. The Journal of Urology *111*, 58-64.

Kozlowski, J., and Grayhack, J. (1991). Carcinoma of the prostate, 2nd edn (Chicago, Mosby Year Book).

Krause, K. H., and Michalak, M. (1997). Calreticulin. Cell *88*, 439-443.

Lee, C., Kozlowski, J., and Grayhack, J. (1995). Etiology of benign prostatic hyperplasia. [Review]. Urologic Clinics of North America *22*, 237-246.

Li, L., Price, J. E., Fan, D., Zhang, R. D., Bucana, C. D., and Fidler, I. J. (1989). Correlation of growth capacity of human tumor cells in hard agarose with their *in vivo* proliferative capacity at specific metastatic sites. Journal of the National Cancer Institute *81*, 1406-1412.

Liu, N., Fine, R. E., Simons, E., and Johnson, R. J. (1994). Decreasing calreticulin expression lowers the Ca2+ response to bradykinin and increases sensitivity to ionomycin in NG-108-15 cells. Journal of Biological Chemistry *269*, 28635-28639.

Mainwaring, W. (1977). The mechanism of action of androgen, Vol 10 (New York, Springer Verlag).

Mery, L., Mesaeli, N., Michalak, M., Opas, M., Lew, D. P., and Krause, K. H. (1996). Overexpression of calreticulin increases intracellular Ca2+ storage and decreases store-operated Ca2+ influx. Journal of Biological Chemistry *271*, 9332-9339.

Mesaeli, N., Nakamura, K., Zvaritch, E., Dickie, P., Dziak, E., Krause, K. H., Opas, M., MacLennan, D. H., and Michalak, M. (1999). Calreticulin is essential for cardiac development. Journal of Cell Biology *144*, 857-868.

Michalak, M., Burns, K., Andrin, C., Mesaeli, N., Jass, G. H., Busaan, J. L., and Opas, M. (1996). Endoplasmic reticulum form of calreticulin modulates glucocorticoid-sensitive gene expression. Journal of Biological Chemistry *271*, 29436-29445.
Michalak, M., Milner, R., Burns, K., and Opas, M. (1992). Calreticulin. [Review]. Biochemical Journal *285*, 681-692.
Nauseef, W. M., McCormick, S. J., and Clark, R. A. (1995). Calreticulin functions as a molecular chaperone in the biosynthesis of myeloperoxidase. Journal of Biological Chemistry *270*, 4741-4747.
Opas, M., Szewczenko-Pawlikowski, M., Jass, G. K., Mesaeli, N., and Michalak, M. (1996). Calreticulin modulates cell adhesiveness via regulation of vinculin expression. Journal of Cell Biology *135*, 1913-1923.
Peterson, J. R., Ora, A., Van, P. N., and Helenius, A. (1995). Transient, lectin-like association of calreticulin with folding intermediates of cellular and viral glycoproteins. Molecular Biology of the Cell *6*, 1173-1184.
Sontheimer, R. D., Nguyen, T. Q., Cheng, S. T., Lieu, T. S., and Capra, J. D. (1995). The unveiling of calreticulin--a clinically relevant tour of modern cell biology. Journal of Investigative Medicine *43*, 362-370.
Vassilakos, A., Michalak, M., Lehrman, M. A., and Williams, D. B. (1998). Oligosaccharide binding characteristics of the molecular chaperones calnexin and calreticulin. Biochemistry *37*, 3480-3490.
Wang, Z., and Brown, D. (1991). A gene expression screen. Proc Natl Acad Sci USA *88*, 11505-11509.
Wang, Z., Tufts, R., Haleem, R., and Cai, X. (1997). Genes regulated by androgen in the rat ventral prostate. Proc Natl Acad Sci USA *94*, 12999-13004.
Zapun, A., Darby, N. J., Tessier, D. C., Michalak, M., Bergeron, J. J., and Thomas, D. Y. (1998). Enhanced catalysis of ribonuclease B folding by the interaction of calnexin or calreticulin with ERp57. Journal of Biological Chemistry *273*, 6009-6012.
Zhou, Z., Wong, C., Sar, M., and Wilson, E. (1994). The androgen receptor: an overview. [Review]. Recent Progress in Hormone Research *49*, 249-274.
Zhu, N., Pewitt, E. B., Cai, X., Cohn, E. B., Lang, S., Chen, R., and Wang, Z. (1998). Calreticulin: an intracellular Ca^{++}-binding protein abundantly expressed and regulated by androgen in prostatic epithelial cells. Endocrinology *139*, 4337-4344.
Zhu, N., and Wang, Z. (1999). Calreticulin expression is associated with androgen regulation of the sensitivity to calcium ionophore-induced apoptosis in LNCaP prostate cancer cells. Cancer Research *59*, 1896-1902.

Appendices: None.